1: Endocrinology 1992 Sep;131(3):1305-12

A new, nongenomic estrogen action: the rapid release of intracellular calcium.

Morley P, Whitfield JF, Vanderhyden BC, Tsang BK, Schwartz JL.

Cell Signals Group, National Research Council of Canada, Ottawa, Ontario.

We have investigated the effects of steroids on the intracellular calcium ion concentration [Ca2+]i in chicken granulosa cells obtained from the two largest preovulatory follicles of laying hens. [Ca2+]i was measured in cells loaded with the Ca(2+)-responsive fluorescent dye fura-2. The resting [Ca2+]i in these cells was 100 +/- 5 nM. There was an immediate (i.e. less than 5 sec) 4- to 8-fold increase in [Ca2+]i in all of the 76 cells examined after the addition of 10(-7) M estradiol-17 bdta. Estradiol-17 beta was effective between 10(-10)-10(-6) M. Estradiol-17 alpha, estrone, and estriol (10(-8)-10(-6) M) were as effective as estradiol-17 beta, but the progestins, pregnenolone, and progesterone, and the androgens, testosterone, androstenedione, or 5 alpha-dihydrotestosterone were ineffective at concentrations up to 10(-5) M. The prompt estradiol-17 beta-induced [Ca2+]i spike was not affected by incubating the cells in Ca(2+)-free medium containing 2 mM EGTA or by pretreating them with the Ca2+ channel blockers lanthanum (1 mM), cobalt (5 mM), methoxyverapamil (D600; 50 microM), or nifedipine (20 microM). The estrogen-triggered [Ca2+]i surge was also not affected by pretreating the cells with the conventional estrogen receptor antagonist tamoxifen (10(-5) M), or the RNA and protein synthesis inhibitors actinomycin D (1 microgram/ml) and cycloheximide (1 microgram/ml), but was abolished by pretreating the cells with inhibitors of inositol phospholipid hydrolysis, neomycin (1.5 mM) and U-73,122 (2.5 microM). The closely related, but inactive, compound U-73,343 (1 microM) did not affect the estrogen-triggered [Ca2+]i surge. Estradiol-17 beta (10(-7) M), but not progesterone (10(-5) M), also triggered a large [Ca2+]i surge in pig granulosa cells, which, like the [Ca2+]i surge in chicken granulosa cells, was almost immediate, transient, and unaffected by incubation in Ca(2+)-free medium or pretreatment with methoxyverapamil (D600; 50 microM), lanthanum (1 mM), or tamoxifen (10(-5)M). However, granulosa cells from immature rats primed with diethylstilbestrol or PMSG did not respond to estradiol-17 beta, even at concentrations as high as 10(-5) M, although they

none of these is art since don't teach that ICI 182780 can differentiate ESR's from nuclear rec's.

-continued

```
AGTTCCCTCT GACTCTTCCC CCACTCCCCA TCTTACTGAT TTAATTTAAT TTTTCACTCC   1020

CCAGAGTCTA ATATGGATTC TGACTCTTAA GTGCTTCCGC CCCCTCACTA CCTCCTTTAA   1080

TACAAATTCA ATAAAAAAGG TGAAATATAA AA                                 1112
```

(2) INFORMATION FOR SEQ ID NO: 98:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1040 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: SPLNNOT10
(B) CLONE: 3339274

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 98 :

```
CGAGCCCNCC CCCAGCGGGA GCTGTGGGGC AGAGGCGCTG CTGTGGTTGG TCAGTCCAGT     60

AAGAAGCCAG CAGGGCTGGG TGCTGGGGCT TCTTCTCCTG AAGGGGCTGC AAGAGGGAAG    120

GCTTAGCCAT GTCGTCCTTG ATCAGAAGGG TGATCAGCAC CGCGAAAGCC CCAGGGGCCA    180

TTGGACCCTA CAGTCAAGCT GTATTAGTCG ACAGGACCAT TTACATTTCA GGACAGATAG    240

GCATGGACCC TTCAAGTGGA CAGCTTGTGT CAGGAGGGGT AGCAGAAGAA GCTAAACAAG    300

CTCTTAAAAA CATGGGTGAA ATTCTGAAAG CTGCAGGCTG TGACTTCACT AACGTGGTGA    360

AAACAACTGT TCTTCTGGCT GACATAAATG ACTTCAATAC TGTCAATGAA ATCTACAAAC    420

AGTATTTCAA GAGTAATTTT CCTGCTAGAG CTGCTTACCA AGTTGCTGCT TTACCCAAAG    480

GCAGCCGAAT TGAAATTGAA GCAGTAGCTA TCCAAGGACC ACTGACAACG GCATCACTAT    540

AAGTGGGCCC AGTGCTGTGT AGTCTGGAAT TGTTAACATT TTAATTTTTA CAATTGATGT    600

AACATCTTAA TTAACCTTTT AATTTTCACA ATTGATGACA GTGTGAGTTT GATGAAAATA    660

TCTGAAGCTA TTATGGAAAT ACCATGTAAT AGGGAGAGTT GAACATGAAT ATTAGAGAAG    720

GAATCCAGTT ACTTTTTTAA ATTACACCTG TGTGCACCTG TATTACTGAA TATAGGAAAG    780

AGATACCCAT TACATAGTTA CTCAGTAAAC AAAAGAGAAA TACCAGGTAG GAAAGAAGAG    840

TTACTATTCC TGAGAAATAA TCAAGAACAT ATTTAATTTA AACTAATGAT GTGAACTATT    900

TAGTTTTGAT GTCCGTTATG TGATTCTGCT TTTACTTGAG TAAAATTAAA GTGTTTAAAT    960

TTGAGATCAA GGAGAAGATA GTGGAACAAA ATGTTATATA GATAATATTT TTCTAATGGA   1020

AATAAAATAG GCAGATTTCC                                              1040
```

What is claimed is:

1. An isolated and purified polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:70, SEQ ID NO:73, SEQ ID NO:79, SEQ ID NO:94, SEQ ID NO:95, and SEQ ID NO:96.

2. An isolated and purified polynucleotide having a nucleic acid sequence which is complementary to the nucleic acid sequence of the polynucleotide of claim 1.

3. A composition comprising the polynucleotide of claim 1.

4. An expression vector containing the polynucleotide of claim 1.

5. A host cell containing the vector of claim 4.

6. A method for producing a polypeptide encoding a human regulatory molecule, the method comprising the steps of:

a) culturing the host cell of claim 5 under conditions suitable for the expression of the polypeptide; and

b) recovering the polypeptide from the host cell culture.

* * * * *

promptly generated a [Ca2+]i transient upon exposure to LHRH (10(-5) M). These results suggest that estrogens almost instantaneously trigger the release of Ca2+ from intracellular stores which may be mediated through phosphoinositide breakdown. The striking rapidity of this estrogen-induced internal Ca2+ mobilization is consistent with the activation of a cell surface receptor which is different from the conventional slowly acting, gene-stimulating nuclear estrogen receptor.

PMID: 1505465 [PubMed - indexed for MEDLINE]

trations of HRM are used to calculate values for the number, affinity, and association of HRM with the candidate molecules.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 98

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 151 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: U937NOT01
(B) CLONE: 133

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1 :

Met Thr Asn Glu Glu Pro Leu Pro Lys Lys Val Arg Leu Ser Glu
5 10 15

Thr Asp Phe Lys Val Met Ala Arg Asp Glu Leu Ile Leu Arg Trp
20 25 30

Lys Gln Tyr Glu Ala Tyr Val Gln Ala Leu Glu Gly Lys Tyr Thr
35 40 45

Asp Leu Asn Ser Asn Asp Val Thr Gly Leu Arg Glu Ser Glu Glu
50 55 60

Lys Leu Lys Gln Gln Gln Gln Glu Ser Ala Arg Arg Glu Asn Ile
65 70 75

Leu Val Met Arg Leu Ala Thr Lys Glu Gln Glu Met Gln Glu Cys
80 85 90

Thr Thr Gln Ile Gln Tyr Leu Lys Gln Val Gln Gln Pro Ser Val
95 100 105

Ala Gln Leu Arg Ser Thr Met Val Asp Pro Ala Ile Asn Leu Phe
110 115 120

Phe Leu Lys Met Lys Gly Glu Leu Glu Gln Thr Lys Asp Lys Leu
125 130 135

Glu Gln Ala Gln Asn Glu Leu Ser Ala Trp Lys Phe Thr Pro Asp
140 145 150

Arg

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 185 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(A) LIBRARY: U937NOT01
(B) CLONE: 1762

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2 :

1: Int Arch Allergy Immunol 1992;98(4):398-409 Related Articles, Books, LinkOut

Estradiol augments while tamoxifen inhibits rat mast cell secretion.

Vliagoftis H, Dimitriadou V, Boucher W, Roznieckí JJ, Correia I, Raam S, Theoharides TC.

Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, New England Medical Center, Boston, Mass 02111.

Mast cells have been studied extensively for their involvement in allergic reactions, where they secrete numerous powerful mediators in response to immunoglobulin E and specific antigens. However, they are also triggered by neuropeptides, they have been found in close contact with neurons, and they are activated in diseases such as angioedema, interstitial cystitis and irritable bowel disease, the prevalence of which is much higher in women. When tested on purified rat peritoneal mast cells, 17 beta-estradiol augmented secretion of histamine and serotonin, starting at 1 microM and in a dose-dependent manner, whether stimulated by the mast cell secretagogue compound 48/80 or the neuropeptide substance P. However, 17 beta-estradiol did not augment mast cell secretion stimulated by immunoglobulin E and specific antiserum indicating that immunologic stimulation is under different regulation. Testosterone inhibited secretion induced by compound 48/80. Tamoxifen, an estrogen receptor antagonist used in the treatment of breast cancer, inhibited serotonin and histamine release from purified rat peritoneal mast cells triggered by compound 48/80 or substance P. Tamoxifen also inhibited the increase in intracellular free Ca2+ originating from an influx of extracellular Ca2+ in response to compound 48/80. Moreover, tamoxifen antagonized the synergistic effect of phorbol myristate and the cation ionophore A23187 on mast cell secretion, suggesting that tamoxifen's inhibition may be due to regulation of protein kinase C activity. Tamoxifen may, therefore, have a beneficial effect in other neuroimmunoendocrine disorders both through estrogen receptor blockade and inhibition of mast cell secretion.

PMID: 1384869 [PubMed - indexed for MEDLINE]

- obj to spec b/o logical contradict.
- LoE of claims.

under increasingly stringent conditions up to 0.1× saline sodium citrate and 0.5% sodium dodecyl sulfate. After XOMAT ARTM film (Kodak, Rochester, N.Y.) is exposed to the blots in a Phosphoimager cassette (Molecular Dynamics, Sunnyvale, Calif.) for several hours, hybridization patterns are compared visually.

VII Microarrays

To produce oligonucleotides for a microarray, SEQ ID NOS:50–98 are examined using a computer algorithm which starts at the 3' end of the nucleotide sequence. The algorithm identified oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that would interfere with hybridization. The algorithm identifies approximately 20 sequence-specific oligonucleotides of 20 nucleotides in length (20-mers). A matched set of oligonucleotides are created in which one nucleotide in the center of each sequence is altered. This process is repeated for each gene in the microarray, and double sets of twenty 20 mers are synthesized and arranged on the surface of the silicon chip using a light-directed chemical process (Chee, M. et al., PCT/WO95/11995, incorporated herein by reference).

In the alternative, a chemical coupling procedure and an ink jet device are used to synthesize oligomers on the surface of a substrate (Baldeschweiler, J. D. et al., PCT/WO95/25116, incorporated herein by reference). In another alternative, a "gridded" array analogous to a dot (or slot) blot is used to arrange and link cDNA fragments or oligonucleotides to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures. A typical array may be produced by hand or using available materials and machines and contain grids of 8 dots, 24 dots, 96 dots, 384 dots, 1536 dots or 6144 dots. After hybridization, the microarray is washed to remove nonhybridized probes, and a scanner is used to determine the levels and patterns of fluorescence. The scanned image is examined to determine degree of complementarity and the relative abundance/expression level of each oligonucleotide sequence in the microarray.

VIII Complementary Polynucleotides

Sequence complementary to the sequence encoding HRM, or any part thereof, is used to detect, decrease or inhibit expression of naturally occurring HRM. Although use of oligonucleotides comprising from about 15 to about 30 base-pairs is described, essentially the same procedure is used with smaller or larger sequence fragments. Appropriate oligonucleotides are designed using Oligo 4.06 software and the coding sequence of HRM, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NOS:50–98. To inhibit transcription, a complementary oligonucleotide is designed from the most unique 5' sequence and used to prevent promoter binding to the coding sequence. To inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding to the transcript encoding HRM.

IX Expression of HRM

Expression of HRM is accomplished by subcloning the cDNAs into appropriate vectors and transforming the vectors into host cells. In this case, the cloning vector is also used to express HRM in *E. coli*. Upstream of the cloning site, this vector contains a promoter for β-galactosidase, followed by sequence containing the amino-terminal Met, and the subsequent seven residues of β-galactosidase. Immediately following these eight residues is a bacteriophage promoter useful for transcription and a linker containing a number of unique restriction sites.

Induction of an isolated, transformed bacterial strain with IPTG using standard methods produces a fusion protein which consists of the first eight residues of β-galactosidase, about 5 to 15 residues of linker, and the full length protein. The signal residues direct the secretion of HRM into the bacterial growth media which can be used directly in the following assay for activity.

X Demonstration of HRM Activity

HRM can be expressed in a mammalian cell line such as DLD-1 or HCT116 (ATCC; Bethesda, Md.) by transforming the cells with a eukaryotic expression vector encoding HRM. Eukaryotic expression vectors are commercially available and the techniques to introduce them into cells are well known to those skilled in the art. The effect of HRM on cell morphology may be visualized by microscopy; the effect on cell growth may be determined by measuring cell doubling-time; and the effect on tumorigenicity may be assessed by the ability of transformed cells to grow in a soft agar growth assay (Groden, J. et al. (1995) Cancer Res. 55:1531–1539).

XI Production of HRM Specific Antibodies

HRM that is substantially purified using PAGE electrophoresis (Sambrook, supra), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols. The amino acid sequence deduced from SEQ ID NOS:50–98 is analyzed using LASERGENEsoftware (DNASTAR Inc) to determine regions of high immunogenicity and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions, is described by Ausubel et al. (supra), and others.

Typically, the oligopeptides are 15 residues in length, synthesized using an Applied Biosystems Peptide Synthesizer Model 431 A using fmoc-chemistry, and coupled to keyhole limpet hemocyanin (KLH, Sigma, St. Louis, Mo.) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel et al., supra). Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity, for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radio iodinated, goat anti-rabbit IgG.

XII Purification of Naturally Occurring HRM Using Specific Antibodies

Naturally occurring or recombinant HRM is substantially purified by immunoaffinity chromatography using antibodies specific for HRM. An immunoaffinity column is constructed by covalently coupling HRM antibody to an activated chromatographic resin, such as CNBr-activated SEPHAROSE (Amersham Pharmacia Biotech). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing HRM is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of HRM (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt antibody/protein binding (eg, a buffer of pH 2–3 or a high concentration of a chaotrope, such as urea or thiocyanate ion), and HRM is collected.

XIII Identification of Molecules Which Interact with HRM

HRM or biologically active fragments thereof are labeled with 1251 Bolton-Hunter reagent (Bolton et al. (1973) Biochem. J. 133: 529). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled HRM, washed and any wells with labeled HRM complex are assayed. Data obtained using different concen-

1: Biochem Biophys Res Commun 1995 Sep 25;214(3):847-55

Estrogen increases endothelial nitric oxide by a receptor-mediated system.

Hayashi T, Yamada K, Esaki T, Kuzuya M, Satake S, Ishikawa T, Hidaka H, Iguchi A.

Department of Geriatrics, Nagoya University School of Medicine, Japan.

To determine the mechanism of the antiatherosclerotic effect of estrogen, we investigated the effect of estrogen on endothelial nitric oxide synthase (NOS-3). Preincubation with a physiologic concentration of 17 beta-estradiol (10(-12)-10(-8) M) over 8 hours significantly enhanced the activity of NOS-3 in endothelial cells of cultured human umblical vein (HUVEC) and of bovine aortas (BAEC). 17 beta-estradiol also enhanced the release of nitric oxide (NO) as measured by an NO selective meter and NO2-/NO3-, metabolites of NO, from endothelial cells. Western blot showed a similar effect of 17 beta-estradiol on NOS-3. The estrogen receptor antagonists, tamoxifen and ICI182780, each inhibited the effect of 17 beta-estradiol by 80%. The effect of 17 beta-estradiol gradually decreased in cells beyond the 10th passage and was not significant in cells beyond the 16th passage. Immunocytochemistry showed the existence of estrogen receptor in HUVEC and BAEC (less than 5 passages) and the sparseness of the existence in BAEC beyond the 16th passage. Estrogen increases NOS-3 via a receptor-mediated system, and estrogen receptor, which appeared to be altered by cell senescence, could be important in the release of NO from endothelium.

PMID: 7575554 [PubMed - indexed for MEDLINE]

Notice of References Cited	Application/Control No. 09/387,372	Applicant(s)/Patent Under Reexamination GABEL ET AL.	
	Examiner Robert Landsman	Art Unit 1647	Page 2 of 2

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
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NON-PATENT DOCUMENTS

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	U	Brenner SE. Trends in Genetics 15:132-133, 1999.
	V	Bork et al. Trends in Genetics 12:425-427, 1996.
	W	Kodym R., et al. Database GenEmbl. Direct Submission. 15 Sept. 1997.
	X	

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6/12/02

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#18	Search **esr1**	13:40:00	105
#11	Search **#6 AND #3**	13:28:26	27
#7	Search **#6 AND #2**	13:25:02	44
#6	Search **#1 AND tamoxifen**	13:22:45	3818
#5	Search **#1 AND #4**	13:22:11	14
#4	Search **#2 AND #3**	13:22:01	1178
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#2	Search **nitric oxide**	13:21:35	48001
#1	Search **estrogen receptor**	13:21:18	21070

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L26	l12 and l16	23	L26
L25	l10 and l19	0	L25
L24	L23 and l16	5	L24
L23	l9 and l19	7	L23
L22	l19 and l19	0	L22
L21	l19 and L20	0	L21
L20	intracellular adj calcium adj levels	305	L20
L19	nitric adj oxide adj release	205	L19
L18	l15 and l16	2	L18
L17	l13 and L16	21	L17
L16	intracellular adj calcium	1608	L16
L15	l13 and L14	3	L15
L14	nitric adj oxide	5571	L14
L13	l6 and L12	193	L13
L12	L11 and human	516	L12
L11	L10 and tamoxifen	534	L11
L10	estrogen adj receptor	1737	L10
L9	estrogen receptor	4343	L9
L8	l7 and tamoxifen	9	L8
L7	l3 and L6	26	L7
L6	l4 or l5	510685	L6
L5	screen or screening	312262	L5
L4	identify or identifying	270830	L4
L3	estrogen with surface with receptor	37	L3
L2	estrogen adj surface adj receptor	0	L2
L1	estrogen surface receptor	2716	L1

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